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Mucopolysaccharide and Protein–Polysaccharide of a Transplantable Rat Chondrosarcoma

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ABSTRACT **Two mucopolysaccharides, chondroitin 4-sulfate (97.8%) and hyaluronic acid (1.2%), were isolated after exhaustive proteolysis of a transplantable chondrosarcoma of the rat. The chondroitin 4-sulfate was fractionated into three fractions of varying degrees of sulfation and chain length. Keratan sulfate and chondroitin 6-sulfate were absent. Extraction of the fresh tumor gave two protein–polysaccharides of similar carbohydrate composition, one soluble in 0.5 M NaCl, the other insoluble. The latter was solubilized in 4 M guanidine·HCl. A dialyzable fraction from the 4 M guanidine solution may be responsible for the insolubility. Both protein–polysaccharides were antigenic and cross-reacted with similar fractions of bovine and human cartilage.**

Human tumors of mesodermal origin have been reported to produce only a single type of mucopolysaccharide, while corresponding normal tissues yield, as a rule, various mucopolysaccharides (1, 2). A study of a transplantable chondrosarcoma in the rat was therefore of interest. Originally, the tumor arose spontaneously in a Sprague-Dawley rat; it has been maintained by subcutaneous implantation for over 5 years in rats of the BUF/N strain. At the 10–12th week after transplantation, the tumors measure at least 40 × 50 × 50 mm and weigh 22–25 g. The tumor persists and grows in noninbred Sprague-Dawley rats, and in strains derived from the Wistar, in addition to the BUF/N rat (R. Swarm).

This preliminary report will describe two sets of experimental data. (*a*) the isolation and properties of the mucopolysaccharides, and (*b*) the isolation and some properties of the protein–polysaccharide of this tumor.

METHODS

Proteolysis and analytical methods were performed as described (3). GlcN and galactosamine (GalN) were determined after hydrolysis with 4 N HCl for 12 hr in an evacuated tube on a Beckman model 120 autoanalyzer. Galactose and xylose were determined on a column of 3% ECNSS-M on a Gas Chrom Q after hydrolysis for 16 hr with 2 N H_2SO_4, passage through ion-exchange columns, reduction with borohydride, and peracetylation (4). Testicular hyaluronidase was purchased from A. B. Leo, Hälsingborg, Sweden, and contained 20,000 IU/mg. It was free of protease as determined viscometrically at 37°C with gelatin as substrate. The gelatin was a purified and lyophilized sample of Gold Label French gelatin.

Abbreviations: KS, keratan sulfate; CPC, cetylpyridinium chloride.

Hyaluronidase digestion was in 0.1 M sodium acetate buffer (pH 5.0) containing 0.15 M NaCl at 56°C for 3 days.

RESULTS

Isolation of mucopolysaccharides

For isolation of mucopolysaccharides, the acetone-dried tissue (6.9% of the wet weight) was exhaustively digested with papain and pronase, followed by alcohol fractionation, a modified cetylpyridinium chloride (CPC)-column fractionation (5), and gel filtration. The isolated material (1.5% of the wet weight) consisted of 97.8% chondroitin 4-sulfate, 1.2% hyaluronate, and 1% of a complex mixture of glycopeptides. Keratan sulfate (KS) was absent, as was evident from the absence of GlcN in the chondroitin sulfate fractions. Hyaluronic acid was characterized by analysis, optical rotation, enzymatic digestion, gel chromatography, and electrophoresis. The chondroitin sulfate fraction proved to be entirely the 4-sulfate isomer, as evidenced by the IR spectrum and by specific chondroitinases and sulfatases (6). The fraction was completely digested by testicular hyaluronidase. CPC fractionation on a cellulose column and gel filtration on an 8% agarose column separated the chondroitin sulfate into three fractions, differing in chemical composition, molecular weight, and sulfate content. The main fraction was fully sulfated and had the highest molecular weight and the lowest peptide content. The two minor fractions were undersulfated and had the lower molecular weight and the higher peptide content. The molar ratio of serine to xylose was fairly constant, while the galactose to xylose ratio was 2.7–2.9, i.e., significantly higher than 2 (7). The chain length, calculated from the ratio of hexosamine to xylose, gave 51.4, 30.1, and 22.2 repeating disaccharide units for the three fractions, corresponding to chain sizes of 25,900, 15,100, and 11,200, respectively. These values agree tolerably well with the chain size calculated from the uronic acid to xylose ratios.

The protein–polysaccharide

The protein–polysaccharide complex of the chondrosarcoma presents some unexpected and puzzling problems, which we believe to be relevant to the general structure of these macromolecules from other sources. Chopped wet tissue was extracted twice at neutral pH in 1.0 N NaCl containing 0.05 M EDTA at a temperature below 0°C. The cleared extract was precipitated with CPC (yield 78% of the total uronic acid). The CPC complex was dissociated in 2 M $CaCl_2$ solution to give 12.8% of the total as a soluble complex (fraction *A*) and the rest as material insoluble in various salts after removal of

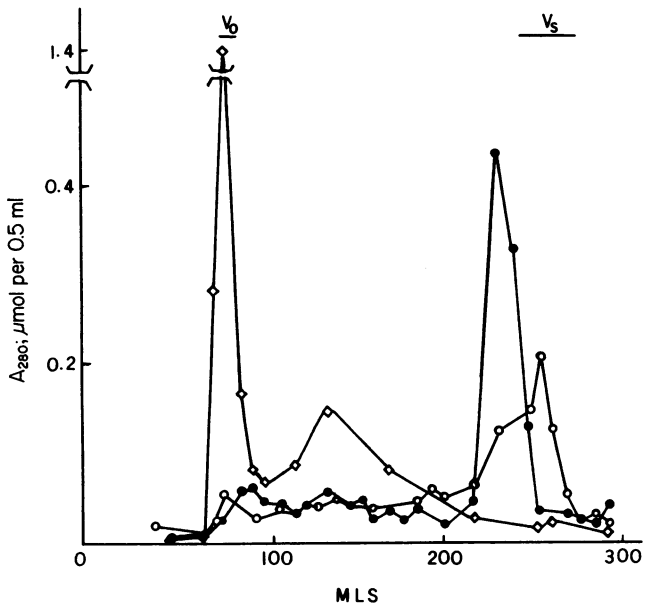

FIG. 1. Elution profile of fraction *A* of chondrosarcoma protein–polysaccharide after exhaustive digestion with testicular hyaluronidase. Fractions were eluted from an 8% agarose column (1.7 × 113 cm) with 0.5 N NaCl solution. ● Anthrone reaction (μmol Gal); O carbazole reaction (μmol GlcUA); ◇A_{280} nm; V_0, void volume (blue dextran 2000); V_s, salt region (Na_2SO_4).

the cetylpyridinium by ethanol precipitation (fraction *B*). Fraction *B* could be solubilized in 6 M urea or 4 M guanidine·HCl. Dithiothreitol was without effect. After precipitation of the 4 M guanidine solution of fraction *B* by ethanol, the fraction was again insoluble in 0.5 M NaCl, while fraction *A* remained soluble after alcohol precipitation. After solubilization of fraction *B* in 4 M guanidine, followed by dialysis or gel filtration, the fraction remained soluble in 0.5 M NaCl even after precipitation with ethanol. Apparently dialysis or gel filtration removes a component that renders the material insoluble. Table 1 shows the analytical data from fractions *A* and *B* after purification by gel filtration. The table shows two unexpected features: (*a*) a relatively high GlcN content, and (*b*) a high ratio of galactose to xylose. With the exception of the higher protein content in *B*, the two fractions are quite similar in composition. Hydroxyproline was absent from both fractions. The serine content of *A* was 68 μmol/g, and of *B* was 188 μmol/g. On alkaline elimination (8) in the presence of borohydride (9), 30% of the serine was lost from both fractions.

TABLE 1. *Analysis of protein–polysaccharides of rat chondrosarcoma*

Component		Preparation *A*	Preparation *B*
Hexosamine	%	18.3	16.7
Glucosamine*/Galactosamine*	%	6.2/93.8	6.4/93.6
Uronic acid	%	23.8	22.0
Protein	%	6.8	16.6
Sulfate/Hexosamine	molar ratio	0.85	0.94
Galactose/Xylose	molar ratio	7.1	5.7
Hexosamine/Xylose	molar ratio	43.8 (22,000)†	47.8 (24,000)†

* Ratio as % of total hexosamine.

† The values in parentheses indicate the chain size (molecular weight).

In order to determine whether the GlcN was present as KS, fraction *A* was digested with a highly purified testicular hyaluronidase and chromatographed on an 8% agarose column (Fig. 1). The protein appeared in two peaks: one in the void volume, the other in a broad peak after the void volume. Both contained small quantities of galactose and uronic acid. The bulk of the oligosaccharide fraction from the hyaluronidase digest, as expected, appeared close to the salt region and overlapped, in part, the main hexose-containing fractions. In these oligosaccharides, about 7% of the total hexosamine was GlcN. This value is similar to that of untreated protein–polysaccharide (Table 1).

Immunological studies* of the two protein–polysaccharide fractions showed one line of precipitation in double-diffusion experiments with antisera prepared in rabbits against protein–polysaccharide of bovine nasal cartilage. Both fractions *A* and *B* injected into rabbits produced antisera that formed a single precipitin line and cross-reacted with human and bovine cartilage protein–polysaccharide; this indicated that they contained common antigenic determinant(s) with the protein–polysaccharides of different species, which are products of normal chondrocytes.

DISCUSSION

The data on the transplantable chondrosarcoma are of interest in several respects. (*a*) The tumor cells presumably originated from a cell arrested in its continued differentiation, since in normal chondrocytes there is a time-related shift for the production of chondroitin 4-sulfate to the 6-sulfate, accompanied by the production of KS (10). (*b*) The chondroitin sulfates produced by the tumor cells are entirely 4-sulfates, while cartilage of other sources yields rarely, if ever, one isomer. Hyaluronic acid, in small quantities, has been found by several laboratories, including our own, in cartilage, especially of young animals. We do not know whether or not it is produced by the malignant chondrocytes. The heterogeneity of the chondroitin 4-sulfate, with respect to chain length and decreased sulfation, likewise may be an expression of a rapid synthesis and discharge by the malignant chondrocytes. (*c*) The absence of KS in the mucopolysaccharide fraction is in accord with the copresence, in normal cartilage and nucleus pulposus, of chondroitin 6-sulfate with KS. (*d*) The separation of the protein–polysaccharide into a soluble and insoluble fraction was unexpected, since by the use of identical procedures on the extractable protein–polysaccharide of chicken xyphoid cartilage (3) and nucleus pulposus (unpublished results) the protein–polysaccharide remained soluble.

* The immunological experiments were performed by Drs. Harold Keiser and John Sandson of the Department of Medicine of the Albert Einstein Medical School, to whom we are greatly indebted. Details of these experiments will be reported in a separate communication. We thank them for permission to report their data.

The insolubility of fraction *B* appears to be related to its content of a dialyzable component and, possibly, to its higher protein content. Whether this dialyzable component is related to the "glycoprotein" fraction described by Hascall and Sajdera (11) and of Rosenberg *et al.* (12) is not known at present.

The nature of the glucosamine-containing portion of the protein–polysaccharide described in this study remains unexplained. Although the molar ratio of GlcN to Gal in protein–polysaccharide (Gal value is corrected for the Gal content of the chondroitin sulfate chains) is 1.62 in fraction *A* and 1.04 in *B*, that is, similar to normal KS, it differs significantly from KS of normal tissues in the following respects: (*a*) No KS was obtained in the mucopolysaccharides isolated after proteolysis. (*b*) The majority of the GlcN (about 90%), and of the hexose-containing portions of the protein–polysaccharide of chondrosarcoma, appear in the salt region on gel filtration (8% agarose column) after exhaustive hyaluronidase digestion, i.e., the GlcN has a molecular size corresponding to an oligosaccharide. In order to exclude the presence of enzyme(s) that might split bonds linking KS to the macromolecules in the sample of testicular hyaluronidase used in this study, a protein–polysaccharide of porcine nucleus pulposus (about 25% KS and 75% chondroitin sulfate) was digested under the same conditions with the identical hyaluronidase preparation. A similar experiment was done with isolated KS of human costal cartilage. In both experiments, no digestion of KS was observed, as shown by the absence of glucosamine in the fractions of low molecular weight. The mechanism of the hyaluronidase action on the GlcN-containing portions of the protein–polysaccharide of chondrosarcoma remains unknown.

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